DECLARATION

I, Yumiko YAMADA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Bldg., 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on May 31, 1999 under Patent Application No. 151769/1999 and that the following is a true and correct translation to the best of my knowledge and belief.

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[File Name] SPECIFICATION

[Title of the Invention] Lyophilized HGF preparation

[Claims]

[Claim 1] A lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent, sodium chloride, and a buffering agent, which is used for preparing an aqueous solution containing the hepatocyte growth factor at a concentration of 5 mg/mL or lower and having a pH and an osmotic pressure desirable as an injection.

[Claim 2] The lyophilized preparation according to claims 1, which is obtained by lyophilizing an aqueous solution containing the hepatocyte growth factor at a concentration of 5 mg/mL or lower.

[Claim 3] The lyophilized preparation according to claim 1 or 2, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides, and a pharmacologically acceptable salt thereof.

[Claim 4] The lyophilized preparation according to any one of claims 1 to 3, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, dextran sulfate, and a pharmacologically acceptable salt thereof.

[Claim 5] The lyophilized preparation according to any one of claims 1 to 4, wherein the buffering agent is a phosphoric acid salt.

[Claim 6] The lyophilized preparation according to any one of claims 1 to 5, which further contains a surface active agent.

[Claim 7] The lyophilized preparation according to claim 6, wherein the surface active agent is a nonionic surface active agent.

[Claim 8] The lyophilized preparation according to claim 7, wherein the nonionic surface active agent is a polyoxyethylene ether surface active agent.

[Detailed Explanation of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a lyophilized preparation comprising a hepatocyte growth factor.

[Prior Art]

Hepatocyte growth factor (abbreviated occasionally as "HGF" hereafter in the specification) is a protein having a proliferating activity of hepatocytes and its

existence in various animal species is known. HGFs having different amino acid sequences have been reported. Human hepatocyte growth factor (abbreviated occasionally as "hHGF" hereafter in the specification) was found from plasma of a fulminant hepatitis patient by Daikuhara et al. (Japanese Patent Unexamined Publication (Kokai) No. 63-22526). The amino acid sequence of the hHGF protein and the gene (cDNA) sequence encoding said protein were found by Kitamura et al. (Japanese Patent Unexamined Publication No. 3-72883). A method for producing the hHGF protein and a transformant using said cDNA have been reported (Japanese Patent Unexamined Publication No. 3-285693). Under the circumstances, mass production of the hHGF protein becomes possible and its application as a medicament is expected.

[0002]

hHGF is a kind of glycoprotein, which is a heterodimer consisting of α subunit having a molecular weight of about 80-90 kDa in a non-reduced state or about 52-56 kDa in a reduced state and β subunit having a molecular weight of about 30-36 kDa. Besides the activity as hepatic cell growth factor, hHGF has various biological activities such as a scatter factor (SF) activity, renal tubular epithelial cell growth factor activity, damaged tissue repair factor activity and vascular endothelial cell growth factor activity, and the protein is expected to be developed as medicaments for therapeutic treatment of liver diseases, kidney diseases, cranial nerve disorders, hair growth promoters, wound healing agents, antitumor therapeutic agents and the like.

[0003]

Pharmaceutical preparations of HGF are described in WO90/10651 and Japanese Patent Unexamined Publication Nos. 6-247872 and 9-25241. The aforementioned WO90/10651 discloses an aqueous preparation of deletion-type HGF (TCF) in which five amino acid residues are deleted from HGF, and the publication teaches that albumin, human serum, gelatin, sorbitol, mannitol, xylitol and the like stabilize TCF in an aqueous solution. Japanese Patent Unexamined Publication No. 6-247872 discloses a preparation containing TCF at a high concentration of 5-10 mg/mL in which a basic amino acid or the like coexists with TCF. This publication refers to the solubility of TCF in an aqueous solution and discloses an aqueous solution containing TCF at a high concentration.

[0004]

However, the aqueous HGF preparation rapidly decreases the solubility of HGF at a neutral pH and has a problem of progress of aggregation, cloudiness and gelation when stored at a low temperature or room temperature for several days. Further, the preparation has low physicochemical stability, for example, formation of degradation products and aggregates, and also has poor stability as a pharmaceutical preparation, for example, reduce of biological activity. Therefore, the preparation is not suitable for a long-term storage from a viewpoint of biological activity. Furthermore, the aqueous HGF preparation may cause aggregation, cloudiness, and gelation due to foaming or the like after shaking and stirring, which leads to decreases of quality of a pharmaceutical preparation and drug efficacy during long-term storage, distribution and transportation. Therefore, a lyophilized preparation is preferred as an HGF preparation.

[0005]

Japanese Patent Unexamined Publication No. 9-25241 discloses a lyophilized preparation of HGF (TCF). The patent publication teaches that a lyophilized preparation of HGF (TCF) that is stable over a long period can be provided by using a citrate as a buffering agent and glycine, alanine, sorbitol, mannitol or the like as a stabilizing agent. However, due to the citric acid used as a buffering agent in the lyophilized preparation, a pH of a redissolved preparation will be 6 or lower in an acidic condition. Further, the resulting solution has a high osmotic pressure, which causes problems of pain at administration by injection, or inflammatory reaction and hemolysis at an administration site and the like.

[0006]

[Objects to be Achieved by the Invention]

An object of the present invention is to provide a lyophilized HGF preparation. More specifically, the object of the present invention is to provide a lyophilized HGF preparation that can be used for preparing an aqueous solution which has a pH and an osmotic pressure ratio desirable as an injection, and has excellent storage stability not to cause aggregation, cloudiness, gelation or the like. Another object of the present invention is to provide a lyophilized preparation that has a favorable cake forming property during lyophilization and excellent solubility, and has excellent long-term storage stability.

[0007]

[Means to Solve the Problem]

The inventors of the present invention conducted various studies to achieve the foregoing objects. As a result, they found that, when a solution containing HGF at a concentration of 5 mg/mL or lower was lyophilized in the presence of a stabilizing agent, sodium chloride and a buffering agent, a lyophilized preparation having a favorable cake forming property, solubility and long-term storage stability was successfully produced. They also found that an aqueous solution having a pH and an osmotic pressure ratio desirable as an injection was successfully prepared from the lyophilized preparation, and the aqueous solution did not cause aggregation, cloudiness, gelation or the like during storage. Further, they also found that the aqueous solution containing the low concentration of HGF successfully exert sufficient clinical effectiveness. The present invention was achieved on the basis of the above findings.

[0008]

The present invention thus provides a lyophilized preparation comprising HGF, a stabilizing agent, sodium chloride and a buffering agent, which is used for preparing an aqueous solution containing HGF at a concentration of 5 mg/mL or lower and having a pH and an osmotic pressure desirable as an injection. This lyophilized HGF preparation can preferably be produced by lyophilizing an aqueous solution containing HGF at a concentration of 5 mg/mL or lower. Examples of pH desirable as an injection usually include usually a range of 6.0 to 7.0, and examples of osmotic pressures desirable as an injection include an osmotic pressure almost isotonic in living bodies or osmotic pressure ratio acceptable as an injection (1 to 2).

[0009]

According to preferred embodiments of the present invention, there are provided the aforementioned lyophilized HGF preparation, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides and pharmacologically acceptable salts thereof; the aforementioned lyophilized HGF preparation, wherein the stabilizing agent is selected from the group consisting of arginine, lysine, dextran sulfate, and pharmacologically acceptable salts thereof; the aforementioned lyophilized HGF preparation, wherein the buffering agent is a phosphoric acid salt; the aforementioned lyophilized HGF preparation, which further contains a surface active

agent; the aforementioned lyophilized HGF preparation, wherein the surface active agent is a nonionic surface active agent; and the aforementioned lyophilized preparation, wherein the nonionic surface active agent is a polyoxyethylene ether surface active agent.

[0010]

[Mode for Carrying Out the Invention]

The type of HGF contained in the lyophilized preparation of the present invention is not particularly limited. For example, natural HGF may be isolated from humors or tissues derived from mammals such as human and rat, which are known to contain HGF, or cells that spontaneously produce HGF. A recombinant HGF obtained by introducing cDNA of said growth factor into cells by gene recombination technique may also be used. Examples of hosts for producing a recombinant HGF include *Escherichia coli*, *Bacillus subtilis*, yeast, filamentous fungi, plant cells, insect cells, animal cells and the like. Specific examples of the recombinant HGF include those obtained from placenta derived from the mammals, liver tissues and blood of a hepatopathy patient, fibroblast strains such as MRC-5 cells and IMR-9 cells, strains producing HGF obtained by introducing an expression vector including cDNA encoding hHGF into a host such as CHO cells according to the method described in Japanese Patent Unexamined Publication No. 3-285693 and the like.

[0011]

Further, as HGF, a precursor protein such as a protein having a signal sequence, a modified protein wherein some of amino acids are replaced, deleted and/or inserted so as not to deteriorate the activity of proliferating hepatocytes, or an altered protein wherein a saccharide is deleted or replaced. Examples of the altered protein include those described in Japanese Patent Unexamined Publication No. 2-288899, WO90/10651, Japanese Patent Unexamined Publication Nos. 3-130091, 3-255096 and 4-30000, Nature, 342, pp.440-443 (1989) and the like.

[0012]

Examples of HGF preferably used for the lyophilized preparation of the present invention include proteinic factors having the following physicochemical properties. The HGF is preferably derived from human. Examples of particularly preferred HGF include those having the amino acid sequences described in Japanese Patent Unexamined Publication Nos. 3-72883 and 4-89499.

1) The factor has an estimated molecular weight of about 76,000-92,000 by SDS-PAGE (under non-reducing condition);

2) the factor has the activity of proliferating hepatocytes; and

3) the factor has strong affinity for heparin.

Further, in addition to the above physicochemical properties, preferred HGF has the following properties:

4) the aforementioned activities are inactivated by a heat treatment at 80°C for 10 minutes; and

5) the aforementioned activities are inactivated by digestion with trypsin or chymotrypsin.

[0013]

HGF has a problem that its solubility is rapidly decreased at neutral pH, since the pH overlaps with the isoelectric point of HGF (pI = 7-8). For example, HGF has low solubility of a little less than 1.0 mg/mL around pH 7.0-7.5 in 10 mM sodium phosphate buffer (PBS, room temperature) containing 140 mM sodium chloride. Whilst HGF has a solubility of 5 mg/mL or higher around pH 5.0, and the solubility of HGF becomes higher at a lower pH. Further, at a sodium chloride concentration of 0.14 M, the solubility of HGF is about 1 mg/mL, and when the concentration is made 0.3 M or higher, HGF is dissolved at a concentration of 5 mg/mL or higher. Therefore, it is necessary that, to increase the solubility of HGF, the solution is kept in an acidic condition at a pH of 5 or lower or the sodium chloride concentration is increased to 0.3 M or higher. However, when a pH of an injection solution is acidified or an osmotic pressure of an injection solution is raised with a high concentration of salts, the solution will cause a pain at an administration by injection or inflammatory reaction and hemolysis at an administration site, which is not preferred.

[0014]

Further, lyophilized HGF preparations containing three ingredients of HGF, a buffering agent and sodium chloride (those described in Japanese Patent Unexamined Publication Nos. 6-247872 and 9-25241: HGF concentration is 5-20 mg/mL) have a problem that, when the content of HGF is reduced to avoid problems such as precipitation of HGF, a favorable cake cannot be obtained in lyophilization process. Further, there is also a problem that aggregation, cloudiness and gelation are observed in an aqueous solution obtained by redissolving a lyophilized preparation obtained

from the above three ingredients, and thus sufficient physicochemical stability cannot be attained. Therefore, to prepare a lyophilized preparation that can give a favorable cake form by lyophilization and enables production of an aqueous solution having excellent long-term storage stability, it is essential to add an additive to improve a cake forming property and storage stability in the state of an aqueous solution.
[0015]

The lyophilized preparation of the present invention is prepared so that an aqueous solution produced from the lyophilized preparation contains HGF at a concentration of 5 mg/mL or lower. The resulting aqueous solution have a pH desirable as an injection and have substantial isotonicity with living bodies or an osmotic pressure ratio acceptable as an injection (1 to 2). Furthermore, the lyophilized preparation of the present invention is characterized to have excellent storage stability. The lyophilized preparation is also characterized in that the preparation can form a favorable lyophilization cake in lyophilization process, and that an aqueous solution obtained by redissolving the lyophilized preparation is free from a problem of aggregation, cloudiness or gelation, thereby sufficient physicochemical stability is achieved. Furthermore, in clinical applications, the preparation can sufficiently exert desired pharmacological actions.
[0016]

Examples of the stabilizing agent include arginine, lysine, histidine, glutamine, proline, glutamic acid, aspartic acid, sulfated polysaccharides such as heparin, chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, and dextran sulfate, and pharmacologically acceptable salts thereof. Examples of the pharmacologically acceptable salts include alkali metal salts such as sodium salts and potassium salts. These stabilizing agents may be used as a combination of two or more kinds. Examples of preferred stabilizing agents include arginine, lysine, dextran sulfate and the like. The amount of the stabilizing agent to be added is not particularly limited as long as the storage stability of HGF can be achieved, but is preferably 0.01-100 times by weight, most preferably 0.1-30 times by weight based on the weight of HGF.
[0017]

The buffering agent is not also particularly limited so long as the agent has an action for adjusting pH of the aqueous solutions before lyophilization and after

redissolution and maintaining solubility of HGF. For example, a phosphate buffer, a citrate buffer, an acetate buffer or the like can be used. As the buffering agent, preferably, a phosphate buffer which has buffering function in almost neutral pH range can be used, and particularly preferably, a sodium phosphate buffer can be used. The amount of the buffering agent to be added is, for example, about 1-100 mM based on the amount of water after redissolution.

[0018]

Sodium chloride improves the solubility of HGF in the aqueous solutions before lyophilization and after redissolution, however, it is not preferred to add sodium chloride more than necessary, because it increases osmotic pressure. In general, it is sufficient to add sodium chloride in an amount sufficient to achieve an isotonic osmotic pressure with living bodies. The osmotic pressure ratio is most preferably 1-2, which is acceptable as the osmotic pressure ratio of an injection. For example, it is preferable to add 140 mM of sodium chloride based on the volume of water after redissolution.

[0019]

The lyophilized HGF preparation of the present invention is preferably added further with a surface active agent. HGF is easily adsorbed to a container material such as glass or a resin. At a low concentration, in particular, adsorption of HGF to a container leads to decrease of a drug content in a solution to be administered. By adding a surface active agent, adsorption of HGF to a container after redissolution can be prevented. Examples of the surface active agent include nonionic surface active agents such as Polysorbate 80, Polysorbate 20, HCO-40, HCO-60, Pluronic F-68 and polyethylene glycol, and a combination of two or more kinds of these agents may also be used. As the surface active agent, polyoxyethylene ether surface active agents (Polysorbate 80 and the like) can be most preferably used. The amount of the surface active agent is, for example, in a range of 0.001-2.0% by weight based on the weight of water after redissolution.

[0020]

The lyophilized HGF preparation of the present invention can be produced by lyophilizing an aqueous solution containing HGF according to a conventional method. For example, HGF, a stabilizing agent, sodium chloride and a buffering agent can be dissolved in distilled water for injection, optionally added with a surface active agent,

sterilized by filtration and introduced into a container such as a vial or an ampoule, and then subjected to lyophilization. The lyophilized HGF preparation of the present invention may contain other additives necessary for formulation, for example, antioxidants, preservatives, excipients, soothing agents and the like. An example of the lyophilization method includes, for example, a method comprising three unit operations: (1) a freezing step for chilling and freezing under atmospheric pressure, (2) a primary drying step for sublimating and drying free water not restrained by a solute under reduced pressure, and (3) a secondary drying step for removing adsorbed water or crystal water intrinsic to the solute (Pharm. Tech. Japan, 8 (1), pp.75-87, 1992). However, the method for producing the lyophilized preparation of the present invention is not limited to the above method. The lyophilized preparation of the present invention can be dissolved by adding a solvent such as distilled water for injection upon use so that the HGF concentration becomes 5 mg/mL or lower.

[0021]

[Examples]

The present invention will be explained more specifically with reference to the following examples. However, the scope of the present invention is not limited to these examples.

Example 1: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF was dissolved at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the conditions shown in Table 1. In the table, "→" indicates that temperature was changed.

[0022]

[Table 1]

	Freezing process	Primary drying process	Secondary drying process

Temperature (°C)	20 → -40	-40	-40 → -20	-20	-20 → 20	20
Time (Hr)	1	5	3	48	2	24
Pressure (mmHg)	760	760	< 1	< 1	< 1	< 1

[0023]

Example 2: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF was dissolved with heating at a concentration of 5 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the conditions shown in Table 1.

[0024]

Example 3: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF was dissolved at a concentration of 10 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the conditions shown in Table 1.

[0025]

Example 4: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF was dissolved at a concentration of 1 mg/mL in 10 mM citrate buffer (pH 5.0) containing 300 mM sodium chloride and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an

amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the conditions shown in Table 1.

[0026]

Example 5: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF was dissolved at a concentration of 1 mg/mL in 10 mM citrate buffer (pH 5.0) containing 300 mM sodium chloride, 5% glycine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the conditions shown in Table 1.

[0027]

Example 6: Preparation of a lyophilized low-concentration HGF preparation (Comparative Example)

HGF was dissolved at a concentration of 1 mg/mL in 10 mM citrate buffer (pH 5.0) containing 300 mM sodium chloride, 5% alanine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the conditions shown in Table 1.

[0028]

Example 7: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

HGF was dissolved at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 100 mM arginine and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the same conditions as in Example 1. Upon use, this preparation was dissolved in 2 mL of distilled water for injection to obtain an injection containing HGF at a concentration of 1 mg/mL and having osmotic pressure ratio (1.5, almost isotonic) acceptable as an injection at almost neutral pH.

[0029]

Example 8: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

HGF was dissolved at a concentration of 1 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the same conditions as in Example 1.

[0030]

Example 9: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

HGF was dissolved at a concentration of 2 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the same conditions as in Example 1.

[0031]

Example 10: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

HGF was dissolved at a concentration of 5 mg/mL in 10 mM phosphate buffer (pH 6.5) containing 140 mM sodium chloride, 100 mM arginine and 0.01% Polysorbate 80 and subjected to filtration for sterilization to obtain an aqueous HGF solution. After pH of the aqueous solution was adjusted, the solution was aseptically charged into vials in an amount of 2 mL per vial. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the same conditions as in Example 1.

[0032]

Example 11: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 6.0) instead of 10 mM phosphate buffer (pH 6.5).

[0033]

Example 12: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 5.5) instead of 10 mM phosphate buffer (pH 6.5).

[0034]

Example 13: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 7.5) instead of 10 mM phosphate buffer (pH 6.5).

[0035]

Example 14: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained by dissolving HGF at a concentration of 1 mg/mL in the same manner as in Example 8 by using 10 mM phosphate buffer (pH 7.0) instead of 10 mM phosphate buffer (pH 6.5).

[0036]

Example 15: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using 50 mM arginine instead of 100 mM arginine.

[0037]

Example 16: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using lysine instead of arginine.

[0038]

Example 17: Preparation of a lyophilized low-concentration HGF preparation (Present

Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using histidine instead of arginine.

[0039]

Example 18: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using glutamine instead of arginine.

[0040]

Example 19: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using cysteine instead of arginine.

[0041]

Example 20: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using proline instead of arginine.

[0042]

Example 21: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using sodium glutamate instead of arginine.

[0043]

Example 22: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using sodium aspartate instead of arginine.

[0044]

Example 23: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using glycine instead of arginine.

[0045]

Example 24: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

A lyophilized low-concentration HGF preparation was obtained in the same manner as in Example 8 by using a charging amount of 5 mL each instead of 2 mL.

[0046]

Example 25: Preparation of a lyophilized low-concentration HGF preparation (Present Invention)

Sodium dextran sulfate was dissolved at concentrations of 50 mg/mL in 10 mM sodium phosphate buffer (pH 6.5) containing 140 mM sodium chloride and 0.01% Polysorbate 80 and then HGF was dissolved at concentrations of 1 mg/mL in this solution. Subsequently, pH was adjusted to obtain an aqueous HGF solution. Then the solution was charged into vials. A lyophilized low-concentration HGF preparation was obtained by lyophilizing the solution according to the same conditions as in Example 1.

[0047]

Test Example 1: Evaluation of solubility of HGF

(1) Method for evaluating solubility of HGF

HGF was weighed in a polypropylene tube and added with 10 mM sodium phosphate buffer containing sodium chloride and a stabilizing agent at various concentrations and 0.01% Polysorbate 80. The tube was immediately maintained at a constant temperature to dissolve HGF. Immediately after the dissolution, the solution was subjected to centrifugation (15,000 rpm, 10 minutes, constant temperature) to completely separate the saturated HGF solution and undissolved HGF. The supernatant was sampled and filtered through a low protein adsorptive filter, Millipore GV (hydrophilic Durapore, 0.22 μ m), and HGF concentration of the resulting saturated solution was quantified by HPLC (gel filtration method) to determine solubility of HGF at saturation.

[0048]

Conditions for HPLC analysis

Column; TOSOH TSK G-3000SWXL (ϕ 0.78 x 30 cm)

Flow rate; 0.3 ml/min

Detection wavelength; OD 280 nm

Temperature;30°C

Carrier; 0.3 M NaCl, 50 mM sodium phosphate, 0.1% SDS, pH 7.5

Application; 50 μ l

Retention time of HGF; 24.0 min

[0049]

(2) Influence of pH on solubility of HGF

Solutions of different pH were prepared by using 10 mM sodium phosphate buffer containing 140 mM sodium chloride and 0.01% Polysorbate 80. The solubility of HGF was examined at 4°C and 20°C by the method of (1). The results are shown in Table 2. The solubility of HGF gradually increased with the decrease of pH. Marked improvement of the solubility was found at pH 5.0 or lower. Further, increase of solubility with elevation of temperature was observed in every sample.

[0050]

[Table 2]

	20°C	4°C
pH 7.5	0.8	0.4
pH 7.0	1.8	1.0
pH 6.0	2.3	1.3
pH 5.0	5.9	4.2

(Solubility of HGF is shown in mg/mL)

[0051]

(3) Influences of sodium chloride concentration on solubility of HGF

10 mM Sodium phosphate buffer solutions (pH 7.5) containing sodium chloride at various concentrations and 0.01% Polysorbate 80 were prepared. The solubility of HGF was examined at 4°C and 20°C by the method of (1). The results are shown in Table 3. Remarkable increase of solubility of HGF was observed with the increase of sodium chloride concentration. Further, increase of solubility with elevation of temperature was observed in every sample.

[0052]

[Table 3]

	20°C	4°C

Not added	0.3	0.1
+ 140 mM NaCl	0.8	0.4
+ 230 mM NaCl	3.2	1.4
+ 300 mM NaCl	8.5	4.0
+ 900 mM NaCl	> 190	-

(Solubility of HGF is indicated in mg/mL)

[0053]

(4) Influence of various stabilizing agents on solubility of HGF

Influence of various additives for pharmaceutical preparations on solubility of HGF was examined. HGF was dissolved at a concentration of 1 mg/mL in 10 mM sodium phosphate buffer solutions (pH 6.8-7.5) containing additives at various concentrations, 140 mM sodium chloride and 0.01% Polysorbate 80 to obtain aqueous HGF solutions. An amount of 200 μ l of each aqueous solution was introduced into each well of a 96-well microtiter plate and stored at 4°C for 48 hours. Then, turbidity of each aqueous HGF solution was determined by measuring OD at 450 nm using a plate reader. The turbidity of the solution increased with the decrease of the solubility of HGF which resulted in aggregation and precipitation of HGF.

[0054]

Influence on HGF solubility was evaluated for additives including 20 kinds of amino acids (arginine, lysine, histidine, serine, threonine, asparagine, glutamine, sodium aspartate, sodium glutamate, cysteine, glycine, proline, alanine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, valine), 7 kinds of saccharides (mannitol, fructose, trehalose, glucose, sorbitol, sucrose, lactose), 3 kinds of polymers (sodium dextran sulfate, dextran, PEG), 3 kinds of proteins (HSA, acidic gelatin, basic gelatin) and 4 kinds of surface active agents (Polysorbate 80, Polysorbate 20, HCO-40, HCO-60). A stabilization effect to maintain the solubility of HGF was observed in the substances listed below.

[0055]

Amino acids: arginine, lysine, histidine, sodium glutamate, sodium aspartate, glutamine, cysteine, proline (the effect was confirmed at 0.05 M),
and polysaccharides: sodium dextran sulfate (the effect was confirmed at 0.1%).

By using the amino acids that gave remarkable effects, solutions were prepared in 10 mM sodium phosphate buffer solutions (pH 7.0) which contained 140

mM sodium chloride, 0.01% Polysorbate 80, and each of the amino acids at a variety of concentrations. The solubility of HGF was examined at 4°C by the method of (1). The results are shown in Table 4.

[0056]

[Table 4]

		Saturation solubility	Osmotic pressure ratio
No additive		1.0	1.0
+ L-Arg	50 mM	7.3	1.3
+ L-Lys	50 mM	4.5	1.3
+ L-His	50 mM	3.2	1.2
+ L-GluNa	50 mM	2.2	1.3
+ L-Arg	100 mM	> 10	1.6
+ L-Lys	100 mM	> 10	1.6
+ L-His	100 mM	4.8	1.4
+ L-GluNa	100 mM	3.2	1.6

(Solubility of HGF is shown in mg/mL)

[0057]

[Effect of the Invention]

A HGF aqueous solution prepared from the lyophilized preparation of the present invention has a pH and an osmotic pressure ratio acceptable as an injection. This solution does not cause precipitation, cloudiness or the like, and can be preserved for a long period of time. Since HGF has a potent efficacy at a concentration of about 0.01-0.1 mg/kg/day in a pathological model and usually a lyophilized preparation contained in a vessel (e.g. vial, ample) is used as a single dosage, a HGF aqueous solution prepared by using the lyophilized preparation of the present invention contains HGF that can exert clinically sufficient therapeutic effectiveness. Further, the lyophilized preparation of the present invention is characterized to have an excellent cake forming property and have excellent long-term storage stability in the state of a lyophilized preparation. The lyophilized preparation is also characterized in that the preparation can be redissolved very easily.

[File Name] ABSTRCT

[Abstract]

[Object] To provide a lyophilized preparation of hepatocyte growth factor that can be used for preparing an aqueous solution which has properties desirable as an injection and does not cause precipitation, cloudiness or the like.

[Means to achieve the object] A lyophilized preparation comprising a hepatocyte growth factor, a stabilizing agent, sodium chloride, and a buffering agent, which is used for preparing an aqueous solution containing the hepatocyte growth factor at a concentration of 5 mg/mL or lower and having a pH (e.g. in a range of 6.0-7.0) and an osmotic pressure desirable as an injection.